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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 33/00 | A1 | (11) International Publication Number: WO 99/44622
(43) International Publication Date: 10 September 1999 (10.09.99)

(21) International Application Number: PCT/GB99/00605

(22) International Filing Date: 1 March 1999 (01.03.99)

(30) Priority Data:
9804469.6 2 March 1998 (02.03.98) GB

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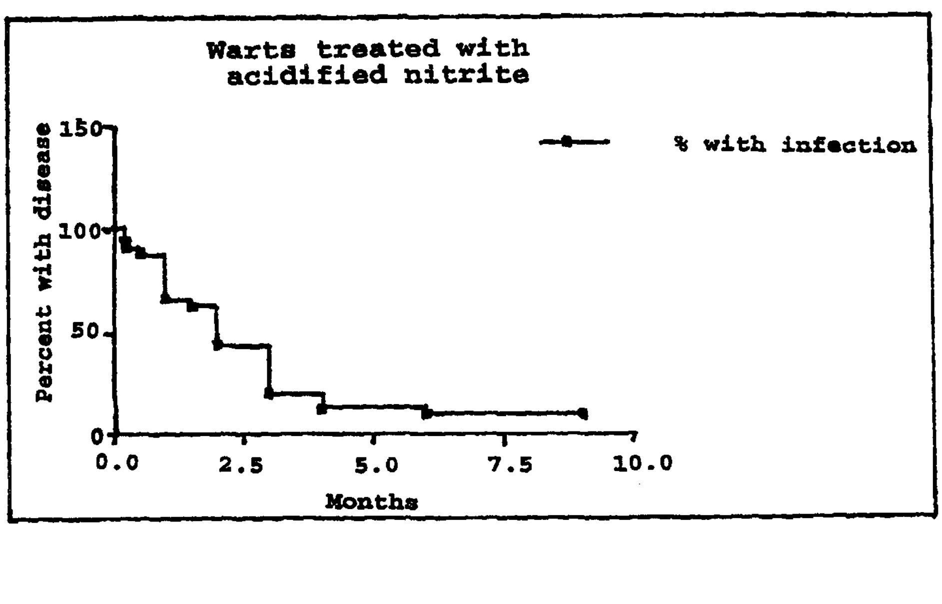
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: INORGANIC NITRITE AND ORGANIC ACID IN COMBINATION AS TOPICAL ANTIVIRAL COMPOSITION



(57) Abstract

The present invention provides for the use in the manufacture of a topical medicament for the *in vivo* potentiation of the immune system during a viral skin infection resultant from virus replication in the epidermis, of a topical formulation comprising a source of nitrogen oxides, characterized in that the source of nitrogen oxides is produced when a pharmaceutically acceptable acidifying agent and a pharmaceutically acceptable donor of nitrogen oxides or a precursor therefor are brought into intimate contact at a site of biological action. The invention also provides delivery systems for the topical medicament.

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INORGANIC NITRITE AND ORGANIC ACID IN COMBINATION AS TOPICAL ANTIVIRAL COMPOSITION

The present invention relates to a complex of nitrogen oxides, arising from the interaction of nitrite and acid as an antiviral composition for the treatment of viral diseases of the skin by topical application thereto. Such nitrogen oxide include particular NO which is of the importance particularly if acidified.

In WO 95/22335 we have disclosed a pharmaceutical composition comprising a pharmaceutically acceptable source of nitrites and a pharmaceutically acceptable acidifying agent, *inter alia* for the direct treatment of disease by topical application. These compounds have a direct effect on the organism concerned but the precise mode of action is not known.

US-A-4595591 reveals a composition comprising an aqueous solution of nitric acid and nitrous acid at a pH below 1 preferably with an organic acid and copper and cadmium ions for the treatment of superficial lesions of the skin, for example tumorous growths.

US-A-5648101 provides a vaso-active composition comprising NO adapted for delivery to a body site *inter alia* by means of a cream or ointment. The NO is generated from an admixture of ferrous sulphate, an organic acid and an inorganic nitrite and caused to be reactive in the presence of moisture adjacent or at the site. Acidification is not discussed.

WO 96/02268 reveals the inhibition of a virus by nitric oxide (NO_2) but the advantages of reduction of pH at the environment of use have not been appreciated.

WO 93/25213 reveals a composition comprising nitrous oxide contained in a dermatological composition comprising as an essential feature a fatty acid or a lower alkyl ester thereof, pH values, particulary at the environment of use, are not mentioned.

The role of NO as a compound which inhibits viral replication *in vitro* has been disclosed by J. B. Mannick; 63rd Forum in Immunology and papers in Intervirology 1995; 38: 206-213, Trends in Microbiology 1995; 3: 81-82, Science 1993; 261:1445-1448, and The Journal of Clinical Investigation 1993; 91: 2446-2452. The above papers disclose the effects of NO on various viruses, for example herpes simplex virus, vaccinia virus and vesicular stomatitis virus. Exogenous NO donors such as S-nitroso-N-acetyl penicillamine (SNAP) or SIN-1 were used *in vitro* to determine the role of NO as an antiviral compound. Application of exogenous NO to cell-lines infected with the virus under test resulted in inhibition of the viral DNA replication. The exact mechanism of the inhibition seemed to differ depending on the virus involved. For example in the case of vaccinia virus it is thought that the NO may inhibit replication by binding to non-haem iron or thiol groups that are essential for the catalytic activity of enzymes involved in vaccinia replication. In this *in vitro* model the antiviral effects of NO do not require immune recognition of infected cells thus providing an early defense against viral pathogens prior to the development of a specific immune response.

In order for viruses to survive and reproduce they must evade recognition by the hosts immune responses. The mechanism by which this is achieved is largely unknown but an effective immune response eradicates the infection. Viruses are

obligate intracellular pathogens. They reproduce using the host's metabolic machinery.

At present drug treatment of viral diseases is predicated upon a small number of compounds which block the replication of the virus. For example Acyclovir, which is effective against herpes virus, is a deoxyguanosine analogue which competes with deoxyguanosine triphosphate as a substrate for viral thymidine kinase and when phosphorylated and incorporated in the viral DNA causes premature DNA chain termination.

Unfortunately anti-viral drugs are only effective for a limited number of viral infections and viruses can mutate to overcome the effectiveness of the drugs. In the case of molluscum contagiosum 1 and 2, which are related to orthopox and parapox viruses and share some homology with vaccinia, other forms of treatment have to be used. Current therapies comprise physical destruction with manual extrusion, liquid nitrogen therapy or curettage, all of which are painful and not very effective and may cause scarring. The pain of these therapies is particularly pertinent because the majority of patients are under the age of 10 years.

In the case of recalcitrant warts, destructive therapies such as liquid nitrogen can be used in cases where the conventional salicylic acid paints have not resulted in the warts disappearance. One problem with warts is that the viral pool is in the stem cells which are found at the base of the epidermis. The aforementioned treatments often remove the virus particles and thus the infection from the top layer of the epidermis, but they do not penetrate deep enough to remove the stem cells and therefore the origins of the infection. This can result in the re-emergence of the warts.

An alternative treatment for warts is by use of dinitrochlorobenzene. Such a treatment is intended to make the patient allergic to dinitrochlorobenzene, whereupon the the patient's immune system mounts an immune response to the dinitrochlorobenzene at the site of the wart and the wart in some cases disappears, presumably as a result of immuno-potentiation. Immuno-potentiation can be an effective treatment but subjecting the patient to an allergic reaction caused by dinitrochlorobenzene can be hazardous variable and difficult to control.

An object of this invention is to provide a treatment for viral skin infections, such as verrucae, warts and molluscum contagiosum, which works effectively and is not associated with the pain involved in the more traditional treatments.

Another object of the invention is to provide a system for treatment of viral skin diseases which is less susceptible to mutation of viral DNA.

We have previously suggested the treatment of viral infected skin with some forms of nitrogen oxides, for example a nitrite and an acidifying agent which results in the following reaction:

$$NO_2^- + H^+ \rightleftharpoons HNO_2 \quad \ldots\ldots\ldots\ldots (1)$$

$$2HNO_2 \rightleftharpoons H_2O + N_2O_3 \quad \ldots\ldots\ldots\ldots (2)$$

$$N_2O_3 \rightleftharpoons NO + NO_2 \quad \ldots\ldots\ldots\ldots (3)$$

$$N_2O_3 + C_6H_8O_6 \rightarrow 2NO + H_2O + C_6H_6O_6 \ldots (4)$$

We have now found that as far as viruses, as opposed to bacteria for example, are concerned, the above nitrogen oxide complex comprising for example NO and/or NO_2 while it may effect replication to a degree, more importantly modifies the virally infected cells such that the immune system can recognize the viral particles. *Inter alia*, this is supported by the fact that the complex is markedly less effective in immunosuppressed hosts. Generally the greater the percent of nitric oxide (NO) the better the immuno potentiation. If possible up to 100% NO can be used.

It is thought, although more work is required, that smaller molecules, particularly NO and NO_2 penetrate the skin by direct diffusion or via the sweat glands or hair follicles through the epidermis to the sweat cells. It has been found that although the healthy keratinocytes find the oxides of nitrogen toxic they do not die as they are relatively resistant to its effects. However, the surprising clinical results in our examples lead us to believe that virally infected cells are more susceptible to these effects, leading to destruction of the virally infected cells via a combination of toxicity leading to programmed cell death and potentiation of the immune response to the presence of the virus.

According therefore to a first aspect of the invention there is provided the use in the manufacture of a topical medicament for the *in vivo* potentiation of the immune system during a viral skin infection resultant from virus replication in the epidermis, of a topical formulation comprising a source of nitrogen oxides, and a pharmaceutically acceptable acidifying agent.

Depending on the type of viral infection the components of the nitrogen oxide source can work synergistically or alone. Nitrogen oxides, for example NO and NO_2, particularly can diffuse through the epidermis. In the case of warts this allows them to reach the stem cells which are at the base of the epidermis and are the cells which contain the pool of established virus. Once at the infected cells the nitrogen oxide complex can facilitate programmed cell death, selectively in infected cells, which may then be taken up by phagocytes and antigen presenting cells leading to immune recognition of the previously hidden viral antigens. Once recognized, specific immunity will lead to destruction of all infected cells through cellular and humoral responses.

Preferably the viruses replicating in the epidermis which cause the viral skin infection are selected from molluscum contagiosum, herpes simplex type 1 and 2, varicella zoster virus and papilloma virus. Treatment using the acidified nitrogen oxide source has been shown to be particularly effective in viral skin infections caused by the aforementioned viruses.

Conveniently the source of nitrogen oxides contains nitric oxide and may also contain NO^- or NO^+ nitrosium ions or a precursor therefor, and is produced when a pharmaceutically acceptable acidifying agent and a pharmaceutically acceptable donor of nitrogen oxides, or a precursor therefor are brought into intimate contact at the site of biological action.

If the pharmaceutically acceptable acidifying agent and the pharmaceutically acceptable donor of nitrogen oxides, or a precursor therefor were brought into contact before reaching the site of biological action the efficacy of the treatment is

diminished as the nitrogen oxides become progressively more inactive with time.

In a preferred embodiment the pharmaceutically acceptable acidifying agent or the pharmaceutically acceptable donor of nitrogen oxides or a precursor therefor is disposed in a pharmaceutically acceptable carrier or diluent therefor.

Preferably the pharmaceutically acceptable acidifying agent is an organic acid and is selected from at least one of ascorbic acid, ascorbyl palmitate, salicylic acid, lactic acid, citric acid, formic acid, benzoic acid and tartaric acid.

The choice of acidifying agent depends on the type of viral infection of the skin and the reaction of the infected areas to treatment. The use of reducing acids such as ascorbic acid gives a quick burst of NO and NO_2 with significantly more NO produced compared to the amount of NO_2 produced. The other organic acids such as salicylic acid give a sustained concentration of NO and NO_2 over a certain time period wherein the ratio of NO to NO_2 is low. The concentration of the inorganic nitrite, for example sodium nitrite (or other alkali metal nitrites), as the pharmaceutically acceptable donor of nitrogen oxides or a precursor therefor depends on the acid used and the concentration of the acid used. The reducing acid ascorbic acid is highly reactive so therefore only between 1-10% is required with stoichiometric concentrations of the pharmaceutically acceptable donor of nitrogen oxides or a precursor therefor (e.g. sodium or other alkali metal nitrite). Ascorbyl palmitate is more stable but requires a higher concentration than ascorbic acid because the palmitate has a higher molecular weight. A concentration of between 3% and 25% of ascorbyl palmitate is thus required. If salicylic

acid is used, concentrations of between 0.5% and 30% are appropriate, citric acid requires a yet higher concentration of up to 45%. (All % given herein are by weight)

The concentration of sodium nitrite required to react with the abovementioned concentrations of organic acid is between 0.5% and 30%, preferably between 5% and 20%. Other pharmaceutically acceptable sources of nitrogen oxides or a precursor therefor require different ranges of concentration.

Preferably the pharmaceutically acceptable acidifying agent and the pharmaceutically acceptable donor of nitrogen oxides or a precursor therefor are in stoichiometric concentrations.

If the pharmaceutically acceptable acidifying agent and the pharmaceutically acceptable donor of nitrogen oxides or a precursor therefor are in stoichiometric concentrations, after the reaction is completed there will be no unreacted compounds left. Accordingly any compounds remaining on the infected area will not be able to contaminate healthy skin with the active medicament or anything the treated area touches such as furniture and clothing.

In a preferred embodiment the medicament is in the form of a paint, a varnish, an ointment a wax, a salve, or a cream. These embodiments allow the pharmaceutically acceptable acidifying agent and a pharmaceutically acceptable donor of nitrogen oxides, or a precursor therefor to be applied directly to the infected area. The treatment comprising the topical application of separate compositions according to this invention is preferably continued for at least one month, and more preferably two months.

In a further aspect of the present invention there is provided a two-part delivery system for the topical application of a medicament for the *in vivo* treatment of the epidermis the said system comprising separately;

a first waxy component comprising a pharmaceutically acceptable acidifying agent;

and a second waxy component comprising a pharmaceutically acceptable source of nitrogen oxides whereby if topically applied *in vivo* simultaneously, or immediately sequentially, to the environment of use, active nitrogen oxides are released therefrom.

In a further embodiment, the first and second waxy components comprise a paraffin. The acidifying agent is preferably a reducing organic acid or salt such as ascorbic acid or ascorbyl palmitate. The source of nitrogen oxides may be an alkali metal nitrite such as sodium nitrite.

The use of a reducing acid or salt thereof results in a product at the environment of use which comprises a major amount of NO which has significant and therapeutic and immunological effects.

Thus of the invention provides for the use of a source of oxide(s) of nitrogen in the manufacture or prophylaxis of a viral skin infection by a virus selected from herpes simplex types 1 and 2, varicella zoster, vaccinia or papilloma, and particularly from molluscum contagiosum.

In a further aspect of the invention there is provided a delivery system for the topical application of a medicament for

the *in vivo* treatment of the epidermis, comprising an adhesive layer and a support layer impregnated with at least one of the components of the medicament, characterized in that the components of the medicament are a pharmaceutically acceptable acidifying agent and a pharmaceutically acceptable donor of nitrogen oxides or a precursor therefor, and a means of combining the pharmaceutically acceptable acidifying agent with the donor of nitrogen oxides. For example whereby the delivery system comprises two layers, which when *in situ* release the oxides of nitrogen including nitric oxide. The activation can be by pressure or hydration from the skin.

Preferably the delivery system is adapted for the potentiation of the immune system during a viral skin infection resultant from virus replication with the delivery system in place, such a system may, for example, resemble an adhesive plaster and it is then simple to apply physical pressure to the exterior of the plaster.

Conveniently the donor of pharmaceutically acceptable nitrogen oxides may be aqueous and encapsulated in microspheres or liposomes disposed in the support, preferably in the form of a film or a gauze. The film or gauze allows increased concentrations of the pharmaceutically acceptable acidifying agent to be used. If a solution of salicylic acid is used then only a concentration of 20- 26% by weight is applied, but if salicylic acid is impregnated in the film or the gauze then a concentration of 26 to 44% by weight can be applied.

A further advantage of using an adhesive layer is that it can be used to occlude the infected area during treatment which increases the concentration of nitrogen oxides being absorbed through the epidermis.

Another advantage of using the delivery system as just described, instead of two creams or ointments, is that the components of the medicament will only be applied to the infected site, i.e no spillage will occur. It is also easier for the elderly, who may have shaky hands, to apply the adhesive layer rather than applying a paint. For the treatment of molluscum contagiosum, which is mainly found in those under the age of 10 years, the adhesive layer can be a decoratively patterned in order to appeal to children.

As stated above preferably the integrity of the vehicle is disrupted by pressure after application of the adhesive layer and film or gauze to a site of viral infected skin. If the pharmaceutically acceptable acidifying agent and the pharmaceutically acceptable nitrogen oxide donors or precursors therefor are not kept separate until administration at the site of biological action they will react together thus rendering the medicament less effective. Accordingly, in this embodiment it is necessary for the pharmaceutically acceptable acidifying agent and the pharmaceutically acceptable nitrites or precursors therefor to be retained separately within the film or gauze layer. The application to the site of biological action of pressure applied to the adhesive layer, and therefore the film or gauze layer, can result in the vehicles, such as the microspheres or liposomes, breaking and the pharmaceutically acceptable acidifying agent and the pharmaceutically acceptable nitrogen oxide donors or precursors therefor reacting, thus treating the infected area.

In another aspect the delivery system may be used in conjunction with a topically applied medicament. The topically applied medicament being either a pharmaceutically acceptable

acidifying agent or a pharmaceutically acceptable donor of nitrogen oxides or a precursor therefor.

It is thus possible to provide only one of either the pharmaceutically acceptable acidifying agent or the pharmaceutically acceptable nitrogen oxide donors or precursors therefor impregnated in the film or gauze layer. The other compound, which is not impregnated in the film or gauze can then be applied topically to the infected site. The advantage of this arrangement is that the film or gauze layer can be larger than the infected site but a reaction between the pharmaceutically acceptable acidifying agent and the pharmaceutically acceptable nitrogen oxide donors or precursors therefor only occurs at the infected site where the medicament had been topically applied.

It is also possible to vary the treatment regime by changing the topically applied medicament without changing the compound in the delivery system. For example if the pharmaceutically acceptable nitrogen oxide donors or precursors therefor are impregnated in the film or gauze, then the type of pharmaceutically acceptable acidifying agent that is topically applied can be altered and the same adhesive and film or gauze layers utilized.

The delivery system is an ideal form of treatment for the verrucae on the feet because the delivery system is hidden from view.

The invention will now be described, by way of illustration only, with reference to the following examples and the accompanying figures.

Figure 1 shows a graph of the duration of the warts compared to the time for wart disappearance with the formulations given in Example 1, where n=32;

Figure 2 shows the outcome of the treatment of patients with warts as a function of time, where n=32

Figure 3 shows a Kaplan Meier plot of the outcome of the treatment of patients with molluscum contagiosum as a function of time; and

Figure 4 shows a graph of NO and NO_2 release from 0.083 g of 10% Ap wax with 0.014 g of 10% sodium nitrite wax to give 21μ moles of $NaNO_2$ and 25μ moles of ascorbyl palmitate. In Figure 4 the curve with "squares" denotes NO values whereas the curve with "circles" denotes NO_2 values.

Example 1

32 subjects with recalcitrant viral warts were treated with varying formulations of sodium nitrite acidified with the acid specified. The exact formulations are given in Table 1. All 32 patients had failed to respond to conventional topical wart applications and at least two treatments with liquid nitrogen. 12 subjects had plantar warts, 12 hand warts, 5 subungal or peri-ungal and 1 plane of the warts of the hand, 1 perianal and 1 lip wart.

The warts had a duration with median 24 months this implies that the patients had a low chance of spontaneous improvement (see Figure 1).

Table 1

Acid	Nitrite	No. of patients treated
Salicylic 5% cream	Sodium nitrite 5% cream	5
Ascorbic acid 5% cream	Sodium nitrite 5% cream	7
Ascorbic acid 10% cream	Sodium nitrite 10% cream	2
Salicylic acid 23% in alcohol based wart paint	Sodium nitrite 10% + copper acetate 0.5%	9
Salicylic acid 23% in alcohol based wart paint	Sodium nitrite 10% cream	3
Salicylic acid 23% in alcohol based wart paint	Sodium nitrite 15% solution	6

The warts were prepared by scraping or abrading the skin to remove the dead skin then the sodium nitrite containing formulation was applied before applying the selected acidifying agent. The warts were treated every night and every three days the warts were rescrapped or abraded.

Clearance of the warts occurred with a median duration of 2 months regardless of the formulation of the treatment (see Figures 1 and 2). Copper was included to catalyze the release of nitrogen oxides from glutathione and proteins that had become nitrosated to extend the release of nitrogen oxides.

Four treatment failures were seen; three of these in patients who were being treated with immunosuppressive drug therapy for Lupus erthematosus, kidney transplant and dermatomyositis. Accordingly there was an 88% cure rate in all the subjects and a 96% cure rate if the immunosuppressed patients were excluded. Existing treatments such as using liquid nitrogen or salicylic acid paints result in 50-80% clearance.

Example 2

30 patients with molluscum contagiosum lesions took part in a double blind trial. They were randomly treated with either 5% sodium nitrite co-applied with 5% salicylic acid under occlusion or 5% sodium nitrite without acidification. The mean age of the subjects was 7 years (with one outlier of 47 not included in the mean). The infection had a mean duration of 8.23±3.959 months. No significant difference was found in the number of lesions per patient or the number of times treatment was applied in the two groups.

In the case of co-application the sodium nitrite was applied to the skin with a cotton bud and then a fresh cotton bud was used to apply the salicylic acid. In the case of the sole application of sodium nitrite it was applied with a cotton bud. In both cases, if possible, the area was covered with "cling-film" or Sellotape®.

As seen in Table 2 in the group treated with the active treatment 70% of the patients were cured and 28% of those in the control group were cured. The mean time to cure was 1.83±0.91 months.

Table 2

Treatment	Cured	Not cured
Acid and Nitrite	12	4
Control	4	10

Kaplan Meier plots were performed for active and control patients (Figure 3) and analyzed by the Logrank test which showed a significant difference in the survival curves with cure being greater in the active group (p=0.0183).

Example 3

12 volunteers with no current or recent history of skin disease and taking no medication randomly applied either low dose (0.5% nitrite) or high dose (5% nitrite) of nitrogen oxide complex to their skin.

Subjects applied 2% w/w ascorbic acid in aqueous cream to a control site and an active site. Either the low dose or the high dose nitrite cream was also applied to the active site. The creams were applied 3 times daily at 8 hourly intervals and both the control and the active sites were then occluded with an adhesive polythene/plastic dressing.

The last application of the cream was made 5 hours before the assessment of the reaction to allow the immediate vasodilatory effects of the nitrogen oxide complex to subside, so measuring only residual inflammation.

The thickness of the control and active sites were measured using a 'Mitotoyu' spring thickness gauge and redness was measured using reflectance erythema metre. Two 4mm punch biopsies were taken from the active and control sites; one for

formalin fixation for histological assessment, mass cell stains, neutrophil elastase and in situ nick end labeling and the other for snap freezing and OCT embedding for the other immunohistochemical stains.

Immunohistochemistry was performed using a streptavidin biotin method and DAB detection with the antibodies in Table 3 and using ApopTag Plus *in situ* nick end labeling detection kit to identify apoptotic cells.

Staining was quantified by computerized image analysis and data analyzed by Wilcoxon's test for paired samples and Kruskal-Wallis' test for non-parametric analysis of variance in the multiple independent samples analyzed for effects of dose and duration (see Tables 4, 5 and 6).

Table 3

Epitope	Titre	Cells Stained
CD1a	0.0451388889	Langerhans cells
CD3	0.0555555556	pan-T cell
CD4	1:150	T-helper cells
CD8	0.0555555556	T-cells suppressor/ cytotoxic
CD54	1:100	ICAM-1
CD6	0.0486111111	Macrophages
CD106	1:100	VCAM-1
p53	0.0763888889	Wild type p53 protein
Nitrosotyrosine *	1:100	Nitrosated tyrosine
Neutrophil elastase	1:100	Neutrophils

Epitope	Titre	Cells Stained
ApopTag **	Manufacturers instructions	Apoptotic cells

* polyclonal, all other antibodies monoclonal

** based on *in situ* detection of cleaved DNA with peroxidase visualization.

The reflectance erythema measurement of the nitrogen oxide complex treated sites was 32.25 ± 5.46 (mean ± sd) significantly higher than the control sites 18.08 ± 5.81 (p=0.0022, Wilcoxon's). Skin fold thickness was 5.04 ± 0.75mm in the nitrogen oxide complex treated patches which was significantly greater than that of control skin 3.25 ± 0.54 (p=0.0022, Wilcoxon's). These measures were not significantly influenced by dose or duration of exposure, except there was a trend for greater skin fold thickness in the high dose group (5.4 mm ± 0.21 vs 4.7 mm ± 0.32)(p=0.075).

Histology of all actively treated sites showed a significant increase in oedema, endothelial swelling, cloudy swelling of keratinocytes, and a mixed infiltrate of lymphocytes and neutrophils. These changes were quantified on a 0-4 ordinal scale and were similar in low dose, high dose, short exposure and long exposure. The number of mast cells seen in Azure A stained sections was similar in control and nitrogen oxide complex treated skin.

A cytotoxic effect was seen in all keratinocytes which was manifest as cloudy swelling. When extensive this leads to the formation of bullae high in the epidermis filled with acute inflammatory cells and cells which have undergone cytotoxic changes with constriction of the nucleus and cloudy swelling

of clear cytoplasm around them. Only a minority of these degenerate cells had undergone apoptosis as judged by staining with ApopTag. Within the viable epidermis, there was also an increase in apoptotic cells. This suggests that normal keratinocytes, not virally infected are relatively resistant to the well known apoptotic effects of nitric oxide. Apoptotic cells were also detected in the dermis, particularly around adnexal structures. The positive nitrosotyrosine staining around sebaceous glands suggests that the nitrogen oxide complex was preferentially absorbed through follicles.

Nitrogen oxide complex treated skin showed significant increases in immuno-competent cells expressing CD3, CD8, CD68 and neutrophil elastase and in the adhesion molecules which attract trafficking of the cells to the site, ICAM-1 AND VCAM-1. The presence of nitrosotyrosine staining in these cells is indicative of the formation of peroxynitrite ($ONOO^-$) and of p53 which indicates that part of the effect of the complex is mediated through toxicity towards DNA in these cells. In healthy skin nitrogen oxide complex did cause some apoptosis but this was surprisingly small at the doses used and we postulate that the effect is likely to be in infected cells. The antigen processing cells of the skin (CD1a positive) were seen to lose dendricity and drop from the epidermis so there were significantly fewer in the treated skin. As these cells behave in this way when activated and functioning to process a newly recognized antigen, this would seem to offer further evidence for an immunopotentiating role for the nitrogen oxide complex. Ki-67 staining for dividing cells did not differ in control or active sites. This would suggest that in warts, for example, the action is not one of reducing cell proliferation.

Kruskal-Wallis test was used to test the effects of time or duration of nitrogen oxide complex treatment on clinical and immunohistochemical response. The effect of the dosage on the skins reaction is given in Table 5. There were fewer CD4 positive cells in the high dose than the low dose group, and likewise with CD68 positive cells. Although Ki-67 positive cells were not significantly different between the control site and the nitrogen oxide complex treated site, there was a significant increase with high dose compared with low dose.

After 24 and 48 hours exposure to the nitrogen oxide complex the extent of apoptosis was measured, see Table 6. There was significantly greater apoptosis after 48 hours than after 24 hours. The CD4 positive cells count rose significantly after 48 hours compared to after 12 hours. The difference for p53 was not quite statistically significant. Similarly, cloudy swelling tended to be greater in the longer duration treatment but was not statistically significant.

Table 4

	Nitrogen Oxide Complex		Control		Significance Wilcoxon's Test
	Mean	S.D.	Mean	S.D.	
ApopTag	12.5	10.1	0.41	1.16	0.0033
Ki67	6.82	3.88	6.62	2.854	0.67
CD1a*	0.86	0.69	3.43	0.53	0.022
CD3	574.7	396.3	216.1	122.1	0.0186
CD4	608.2	458.2	176.3	149.9	0.0125
CD8	275.7	193.1	122.1	106.1	0.0284
CD68	673.1	542.4	301.4	361.3	0.0044

	Nitrogen Oxide Complex		Control		Significance Wilcoxon's Test
Nitrosotyrosine *	3.4	0.7	0.9	1.1	0.043
p53	214.4	266.4	22.08	53.8	0.0029
Neutrophils	569.4	385.9	71	113.1	0.043
ICAM-1	705.9	704.5	201.9	160.9	0.0209
VCAM-1	1.5	1.17	0.5	0.9	0.0357

* Where cell counting was difficult e.g. more diffuse staining/dendritic cells, staining was graded subjectively on a scale of 0-4.

Ki67 was counted in the epidermis and ApopTag positive cells counted per standard section through a 3mm punch biopsy. All other counts were done by computerized image analysis on a fixed standard measuring frame and are expressed as cells per mm^2.

Table 5

	High Dose (cells/mm^2)		Low Dose (cells/mm^2)		Kruskal-Wallis Test
	Mean	S.D.	Mean	S.D.	
Ki67	152	35.2	73.6	8	0.01
CD4	280	78.4	936	185.6	0.02
CD68	379.2	65.6	916.8	262.4	0.04

Table 6

	12/24* hrs		48 hrs		Kruskal-Wallis Test
	Mean	S.D.	Mean	S.D.	
ApopTag *	3.5	1.82	14.16	2.56	<0.005

CD4	285.9	193.6	824	193.6	0.05
Cloudy swelling **	1.7	0.33	2.5	0.224	0.07
p53	70.1	47.04	335.2	125.7	0.07

Ki67 was counted in the epidermis and ApopTag positive cells counted per standard section through a 3mm punch biopsy. All other counts were done by computerized image analysis on a fixed standard measuring frame and are expressed as cells per mm^2.

** Where cell counting was difficult e.g. more diffuse staining/dendritic cells, staining was graded subjectively on a scale of 0-4.

The nitrogen oxide complexes of the invention may be formed by a combination of ascorbic acid and nitrite on the skin, which causes the release of nitrogen oxides, *inter alia* nitric oxide, nitrous oxide, nitrogen dioxide and dinitrogen trioxide. The increase in T helper cells and macrophages was greater in low dose subjects and suggests that at lower doses nitrogen oxides can be pro-inflammatory but at higher doses becomes cytotoxic to the immunocompetent cells and begins to exert an inhibitory effect. The nitrogen oxide complex led to a marked induction of ICAM-1 and a moderate increase in VCAM-1 expression. The pattern of inflammation was unusual in showing a marked infiltrate of macrophages after only 24 hours, so showing that activated macrophages use nitrogen oxides to specifically attract more macrophages to kill a pathogen.

The promotion of apoptosis and recruitment of all the immunocompetent cells required for effective recognition of a

pathogen by the immune system of a host, results from application of a preparation of a combination of nitrite or precursor of nitrogen oxides and an acidifying agent. Accordingly, these findings support a potential immunopotentiating effect of the combination of nitrite or other precursor of nitrogen oxides such as NO or NO_2 and a acidifying agent.

Example 4

A two part component delivery system was made up. Each component was in the form of a wax stick which can be rubbed onto an effective area at regular intervals in accordance with a physician's instructions.

The two components were made up as follows:-

10% ASCORBYL PALMITATE

Component	
Ascorbyl Palmitate	10%
White Soft Paraffin	25
Light Liquid Paraffin	20
Hard Paraffin	20
Arlacel 165	15
Cetosteryl Alcohol	10

Method

1. Weigh all the components into a vessel.
2. Heat the vessel and stir the mixture until all the components have melted and the mixture is homogenous.
3. Pour the molten wax into jars and allow to cool to room temperature.

10% SODIUM NITRITE WAX

Components	
Phase A	
Light Liquid Paraffin	7.5%
White Soft Paraffin	20
Arlacel 582	10
Cetosteryl alcohol	10
Phenoxyethanol	1
Phase B	
Sodium Nitrite	10
Purified Water	20

Method

1. Weigh the Phase A components into a vessel, heat to 70°C and stir until homogenous.
2. Weigh the Phase B components into another vessel heat to 70°C and stir, ensure that all the sodium nitrite has dissolved.
3. When both phases have reached 70°C, add phase A to phase B and homogenize for 5 minutes.
4. Pour the molten wax into jars and allow to cool to room temperature.

As is shown from Figure 4, the use of this admixture tends to release a substantial excess of NO from the two-part delivery system. This is possibly because NO is a small molecule which results in a more effective treatment of viral skin diseases.

CLAIMS:-

1. The use in the manufacture of a topical medicament for the *in vivo* potentiation of the immune system during a viral skin infection resultant from virus replication in the epidermis, of a topical formulation comprising a source of nitrogen oxides, characterized in that the source of nitrogen oxides is produced when a pharmaceutically acceptable acidifying agent and a pharmaceutically acceptable donor of nitrogen oxides or a precursor therefor are brought into intimate contact at a site of biological action.

2. The use according to claim 1 where the nitrogen oxides are substantially nitric oxide *per se*.

3. The use according to claim 1 or 2 wherein the viral skin infections are selected from molluscum contagiosum, herpes simplex type 1 and 2, varicella zoster virus and papilloma virus.

4. The use according to any of claims 1 to 3 wherein the acidifying agent is also a reducing agent.

5. The use according to any preceding claim wherein the acidifying agent is such that when applied at an environment of use, the pH is reduced below 5 but above 2.

6. The use according to any preceding claim wherein the pharmaceutically acceptable acidifying agent is an organic acid.

7. The use according to any preceding claim wherein the pharmaceutically acceptable nitrogen oxide donor or a precursor therefor is an inorganic nitrite.

8. The use according to any preceding claim wherein the pharmaceutically acceptable acidifying agent is selected from ascorbic acid, ascorbyl palmitate, salicylic acid, lactic acid, citric acid, formic acid, benzoic acid and tartaric acid.

9. The use according to claim 7 wherein the pharmaceutically acceptable nitrogen oxide complex or precursor therefor is an alkali metal nitrite or a precursor therefor.

10. The use according to claim 8 wherein ascorbic acid is present in a concentration between 1-10% by weight and the concentration of the alkali metal nitrite or a precursor therefor is 0.5-30% by weight.

11. The use according to claim 8 wherein the concentration of salicylic acid is between 0.5%-30% by weight and the concentration of the alkali metal nitrite or a precursor therefor is 0.5-30% by weight.

12. The use according to claim 8 wherein the concentration of citric acid is between 0.5%-45% by weight and the concentration of the alkali metal nitrite or a precursor therefor is 0.5-30% by weight.

13. The use according to claim 8 wherein the concentration of ascorbyl palmitate is between 3%-25% by weight and the concentration of the alkali metal nitrite or a precursor therefor is 0.5-30% by weight.

14. The use according to claim 8 wherein the concentration of lactic acid is between 0.5-20% by weight and the concentration of the alkali metal nitrite or a precursor therefor is 0.5-30% by weight.

15. The use according to claim 8 wherein the concentration of formic acid is between 0.5-20% by weight and the concentration of the alkali metal nitrite or a precursor therefor is 0.5-30% by weight.

16. The use according to claim 8 wherein the concentration of benzoic acid is between 0.5-20% by weight and the concentration of the alkaline metal nitrite or a precursor therefor is 0.5-30% by weight.

17. The use according to claim 8 wherein the concentration of tartaric acid is between 0.5-20% by weight and the concentration of the alkaline metal nitrite or a precursor therefor is 0.5-30% by weight.

18. The use according to any preceding claim wherein the pharmaceutically acceptable acidifying agent and the pharmaceutically acceptable source of nitrogen oxides or a precursor thereof are in stoichiometric concentrations.

19. The use according to any of the preceding claims wherein the medicament is in the form of at least two separately disposed active ingredients disposed in an ointment, a cream, a wax, a varnish or a paint.

20. The use accordingly to any preceding claim wherein the topical medicament is adapted to be applied for at least one month.

21. The use of a source of acidified oxide(s) of nitrogen in the manufacture of a topical medicament for the treatment or prophylaxis of a viral skin infection by a virus selected from molluscum contagiosum, herpes simplex types 1 and 2, varicella zoster, vaccinia or papilloma.

22. The use according to claim 21 wherein the virus is molluscum contagiosum.

23. A delivery system for the topical application of a medicament for the *in vivo* treatment of the epidermis, comprising an adhesive layer and a support layer impregnated with at least one of the components of the medicament characterized in that the components of the medicament are a pharmaceutically acceptable acidifying agent and a pharmaceutically acceptable source of nitrogen oxides or a precursor therefor and a means for combining the pharmaceutically acceptable acidifying agent with the source of nitrogen oxides.

24. A delivery system according to claim 23 wherein the delivery system comprises microspheres.

25. A delivery system according to claim 23 wherein the delivery system comprises liposomes.

26. A delivery system according to any of claims 23 to 25 wherein the delivery system is used in conjunction with a topically applied component.

27. A delivery system according to claim 26 wherein the topically applied component is either a pharmaceutically acceptable acidifying agent or a pharmaceutically acceptable source of nitrogen oxides or a precursor therefor.

28. A delivery system according to either of claims 26 or 27 wherein the topically applied component is in the form of an ointment, a cream, a varnish, a wax, a salve, or a paint.

29. A delivery system according to any of claims 23 to 28 wherein the pharmaceutically acceptable acidifying agent is selected from ascorbic acid, ascorbyl palmitate, salicylic acid, lactic acid, citric acid, formic acid, benzoic acid and tartaric acid.

30. A delivery system according to any of claims 23 to 29 wherein the concentration of salicylic acid contained within the microsphere is between 0.5%-45%.

31. A two-part delivery system for topical application of a medicament for the *in vivo* treatment of a viral infection of the epidermis, said system comprising separately;
(a) a first waxy component comprising a pharmaceutically acceptable acidifying agent; and
(b) a second waxy component comprising a pharmaceutically acceptable source of nitrogen oxide, whereby if topically applied simultaneously or immediately sequentially to an environment of use active nitrogen oxides are released thereto.

32. A delivery system according to claim 31 wherein the first and second waxy component comprise a paraffin.

33. A delivery system according to either of claims 31 or 32 wherein the acidifying agent is a reducing organic acid or salt.

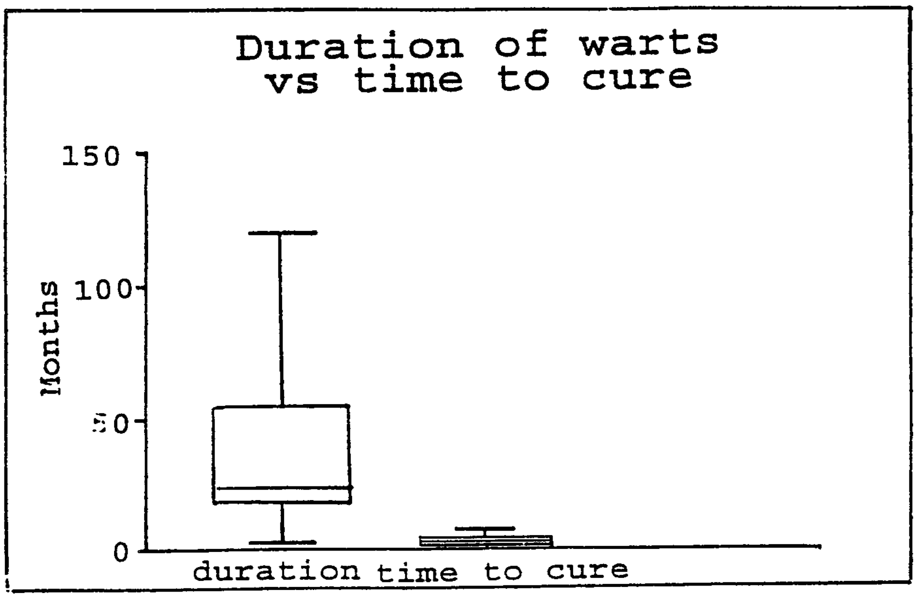

Duration of warts
vs time to cure
150
100
50
0
Months
duration
time to cure

Figure 1

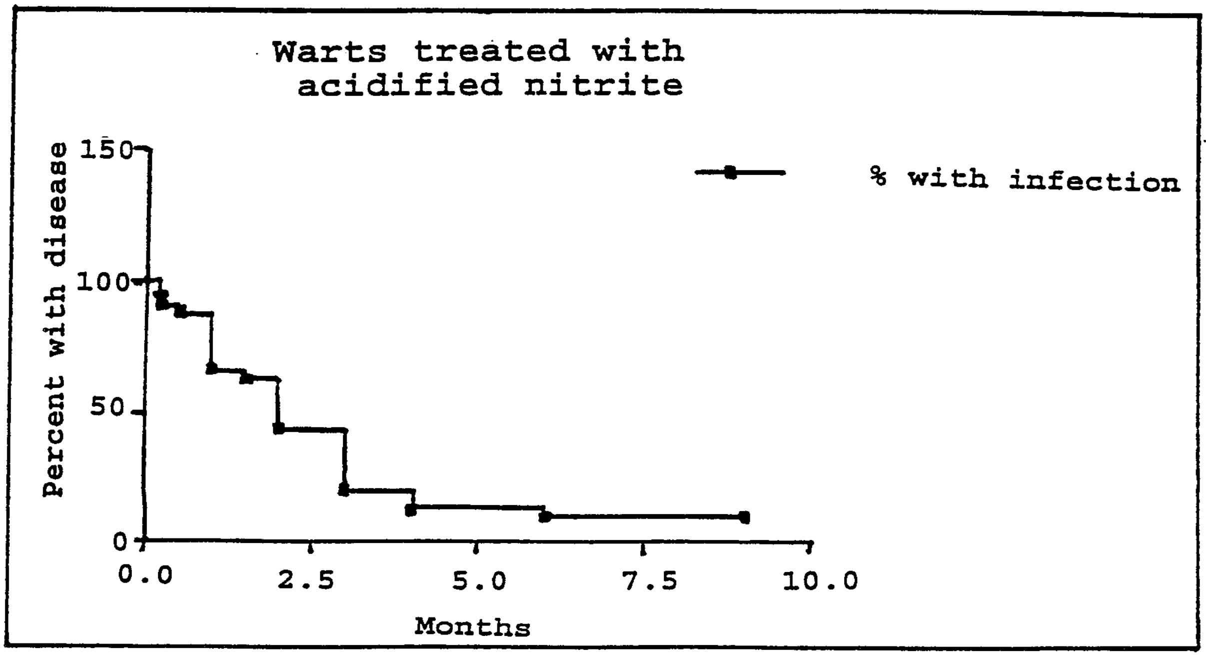

Warts treated with
acidified nitrite
% with infection
Percent with disease
150
100
50
0
0.0
2.5
5.0
7.5
10.0
Months

Figure 2

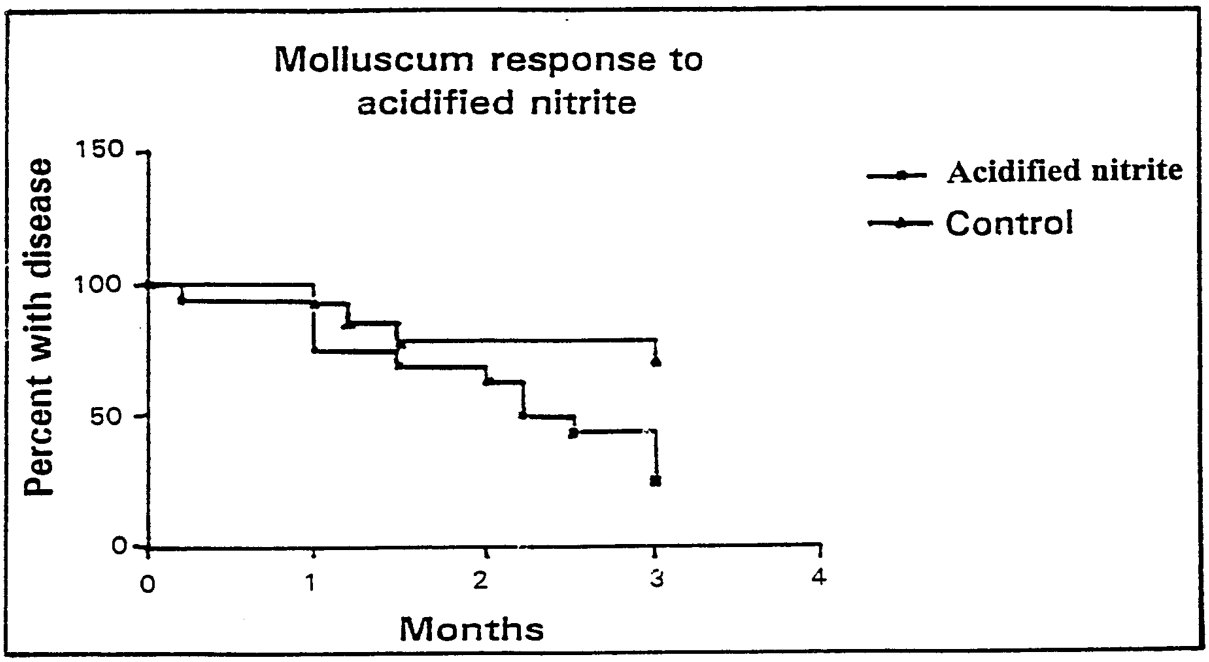
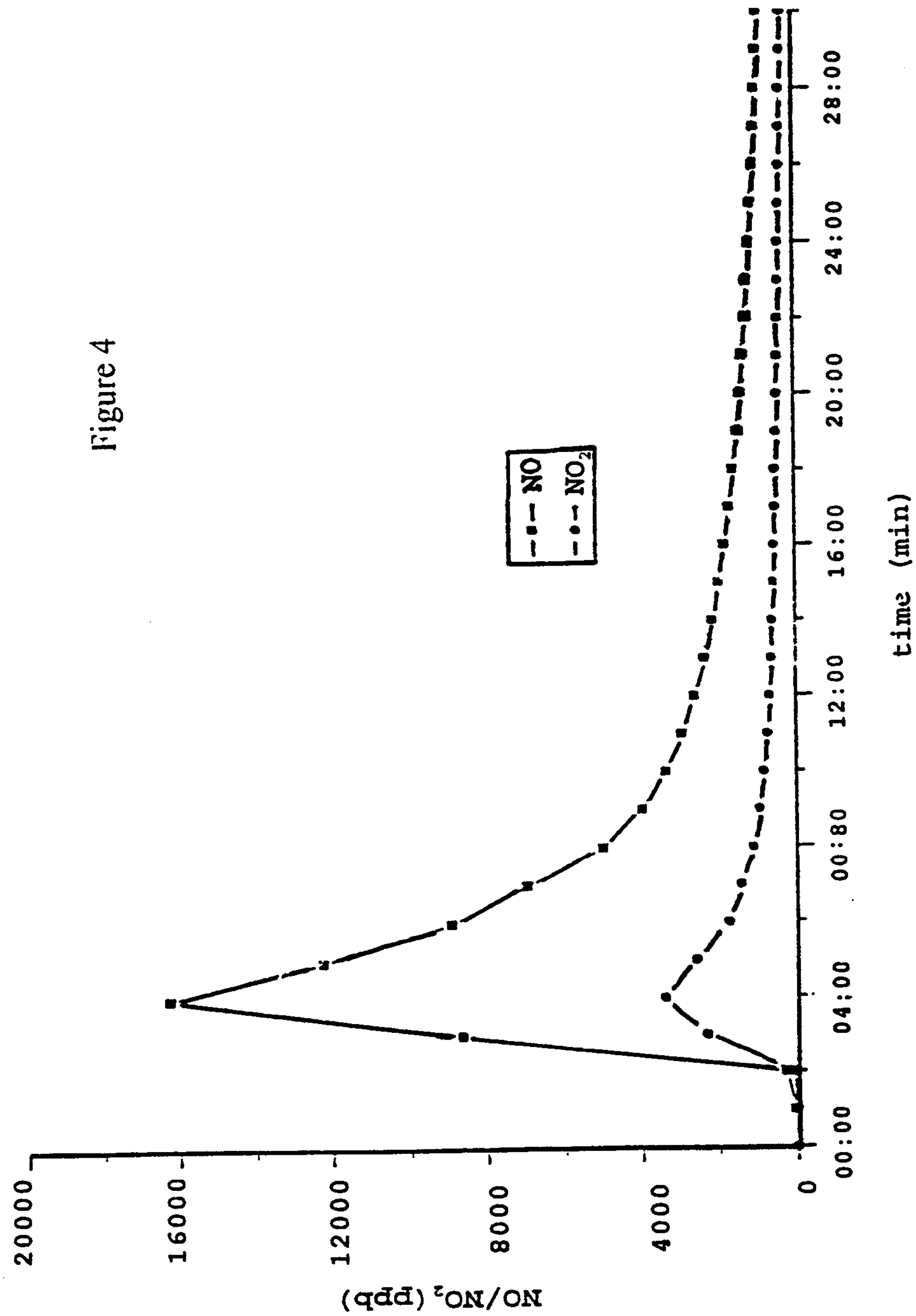

Molluscum response to acidified nitrite
Percent with disease
150
100
50
0
0
1
2
3
4
Months
Acidified nitrite
Control

Figure 3

Figure 4


NO
NO2
NO/NO2 (ppb)
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16000
12000
8000
4000
0
00:00
04:00
08:00
12:00
16:00
20:00
24:00
28:00
time (min)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/00605

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K33/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 4 923 899 A (WACHMAN STANLEY L ET AL) 8 May 1990 (1990-05-08) abstract; claim 1; examples 4,8 ---	1,2,5-7, 9,18,20
X	US 5 573 786 A (GRABO MICHAEL ET AL) 12 November 1996 (1996-11-12) abstract column 2, paragraph 1; claims 4-7; examples 5,9,10 ---	1,2,5-7, 9,18,20
X	WO 95 22335 A (UNIV ABERDEEN ;BENJAMIN NIGEL (GB); DOUGALL HAMISH (GB)) 24 August 1995 (1995-08-24) cited in the application abstract; claims 1-5; example 5 --- -/--	1-33

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
10 August 1999	19/08/1999

Name and mailing address of the ISA	Authorized officer
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1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Information on patent family members

International Application No
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